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PRINCIPAL INVESTIGATOR: Chin-Rang Yang, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14627

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Chin-Rang Yang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester
Rochester, New York 14627

E-Mail: chinrang_yang@urmc.rochester.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The formation of DNA double strand breaks (DSBs) correlates well with lethality of cancer cells following ionizing radiation (IR). The DNA-dependent protein kinase (DNA-PK) complex is a major factor in the repair of DSBs in mammalian cells. It is composed of a catalytic subunit (DNA-PKcs) and a DNA end binding subunit (the Ku70/Ku80 heterodimer). Given Ku70 knock-out mice show both hypersensitive to IR and growth retardation, Ku70 are involved in DNA repair and cell growth. Blocking Ku70 function can have dual impacts on cancer cells: to sensitize cancer cells to DNA damage agents, such as ionizing radiation and chemotheraputic drugs, while suppress cancer cell growth.

We confirmed Ku70-Hsp90 interaction in yeast and human breast cancer cells, and is needed for Hsp90 stability. Hsp90 is a molecular chaperone, and its client proteins are typically involved in signal transduction (Akt), cell cycle control. Ku70 antisense oligo treatment lowers protein levels in both Hsp90 and Akt similar to Hsp90 inhibitors suggesting Akt signal pathway is the downstream of Ku70-Hsp90 interaction for cell growth. Overall, the research supported by this grant not only lead to better understanding of Ku70-Hsp90 related-cell growth pathway, but also to identify Ku70-Hsp90 as a new target for cancer therapy.

14. SUBJECT TERMS
DNA repair, cell signaling, Ku70, Hsp90, Akt, breast cancer

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Table of Contents

PI: Yang, Chin-Rang

Inhibition of DNA-Dependent Protein Kinase Activity for Breast Cancer Therapy

Introduction:

The formation of DNA double strand breaks (DSBs) correlates well with lethality of cancer cells following ionizing radiation (IR)(1). The DNA-dependent protein kinase (DNA-PK) complex is a major factor in the repair of DSBs in mammalian cells. It is composed of a catalytic subunit (DNA-PKcs) and a DNA end binding subunit (the Ku70/Ku80 heterodimer). Given Ku70 knock-out mice show both hypersensitive to IR and growth retardation, Ku70 are involved in DNA repair and cell growth (2,3). We hypothesize that blocking Ku70 function can have dual impacts on cancer cells: to sensitize cancer cells to DNA damage agents, such as ionizing radiation and chemotheraputic drugs, while suppress cancer cell growth. The purpose of this research is to identify factors that involve in the Ku70 mediated cell growth pathway as targets for future cancer therapy.

Body

A. Isolation of Ku70 associated proteins:

Isolation of Ku70 associated proteins by yeast two-hybrid cloning with various cDNA libraries.

The similar yeast two-hybrid cloning with Ku70 as bait using human bone marrow, ovary, placenta and brain libraries have been performed at P.I. laboratory at the University of Rochester. Results are summarized in Table 1.

Table 1: Ku70-associated Proteins by Yeast Two-hybrid Analysis

	Gene	Human cDNA library
1.	Ku80	Liver, Bone Marrow, Ovary
2.	Clusterin	Liver, Bone Marrow, Ovary
3.	Heat Shock Protein 90	Bone Marrow
4.	unknown	Liver, Bone Marrow, Ovary

Table 1, Ku80 and clusterin were isolated again from human bone marrow (BM) and ovary libraries in Ku70 clonings as expected. The new Ku70 associated protein is heat shock protein 90 (Hsp90) from bone marrow library.

Table 2: Specificity of Ku70-Hsp90 Interaction

	Hsp90	Hsp70
Ku70	++	--
Ku70 StuI	--	--
Ku80	--	--
p53	--	--
cdk2	--	--
lamin B	--	--
SNF-1	--	--

Table 2 Hsp90 interacts specifically with Ku70, not with Ku70 StuI (a mutant Ku70 with c-terminus deletion), Ku80, p53, cdk2, lamin B and SNF-1. The other heat shock protein (Hsp70) dose not interact with Ku70. Thus, we conclude that Ku70 and Hsp90 interaction is specific in the yeast two-hybrid system.

B. Interaction of Ku70 and Hsp90:

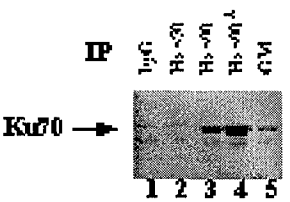
The eukaryotic Hsp90 is a molecular chaperone and its client proteins are typically involved in signal transduction, cell cycle control, or transcriptional regulation (4,5). Ku70 and Hsp90 interaction is also confirmed by co-immuoprecipitation, and co-localization using confocal microscopy. Since Ku70 is primarily located in nuclei and Hsp90 is primarily located in cytoplasm, we theorize that these two proteins may be met in

(1) late G2/M and early G1 phases of cell cycle when the nuclear membrane is not intact;

(2) Hsp90 may be a chaperone for new synthesized Ku70 during nuclear translocation;

(3) nuclei are damaged in pathological condition, drugs treatment, or ionizing radiation treatment.

A.



B.

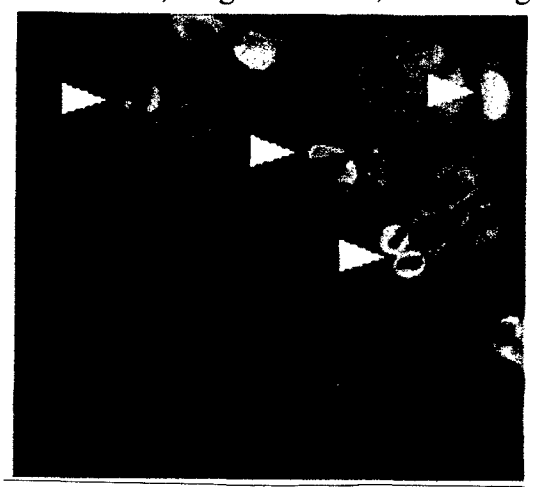


Figure 1. Ku70-Hsp90 Interaction in mammalian cells. Human breast cancer MCF-7 cells were used in these experiments. **(A)** Lane 1: cell lysate were immunoprecipitated using normal IgG as negative controls; Lane 2: cell lysate were immunoprecipitated using Hsp70 antibody as negative controls; Lane 3: cell lysate of normal growing cells without any treatment was immunoprecipitated using Hsp90 antibody; Lane 4: cell lysate with colcemid treatment were immunoprecipitaed using Hsp90 antibody;

Lane 5: cell lysate with Geldanamycin (GM, a Hsp90 inhibitor) treatment were immunoprecipitaed using Hsp90 antibody.

(B) Cells were fixed and immuno-stained with both Ku70 and Hsp90 antibodies. Ku70 antibody was detected by secondary antibody with Texas red and Hsp90 antibody was detected by secondary antibody with FITC (green). Co-localization of these two proteins was visualized by confocal microscopy and shown in yellow color (white arrowheads).

We first treated MCF-7 cells with colcemid (100ng/ml) for 18 hours, and larger than 80% of cells are in G2/M phase judged by flow cytometry. Cell lysates were harvested and co-immunoprecipitaion was performed using either Hsp90 antibody. In Figure 1A, lane 4 indicates that more Ku70/Hsp90 complexes were formed when cells are in G2/M phase than untreated cells (lane 3). Normal IgG (lane 1) and Hsp90 antibody (lane 2) immunoprecipitation did not bring down Ku70 served as negative controls for the specificity of Ku70-Hsp90 interaction. Geldanamycin (GM, a Hsp90 inhibitor) block the interaction of Ku70 and Hsp90 (lane 5).

In Figure 1B, most of cells show distinct distribution of Ku70 (in nuclei, red) and Hsp90 (in cytoplasm, green). However, cells in M-phase of cell cycle show colocalization of these two proteins (indicated by white arrowheads). To further understand the function of Ku70-Hsp90 interaction in Ku70 mediated cell growth pathway, we decide to use Ku70 antisense oligo to block Ku70 protein expression in mammalian cells and examine effects on Hsp90 and its client proteins.

C. Ku70 antisense treatment:

Design of antisense oligo

Antisense oligonucleotides function by hybridizing to their target mRNA; thereby, blocking translation. Translation is inhibited by either degradation of the transcript by RNase H or L at the RNA:DNA duplex, or by blocking the ribosome thus subsequently inhibiting protein synthesis. Benefits of antisense oligo including sequence specific inhibition of gene expression, faster and cheaper than knockout mice experiments. However, due to the secondary structure of mRNA, designing an effective antisense oligo is a challenge. By using the guidelines provided below, P.I. is able to obtain an effective antisense oligo right away. Most importantly, investigators can use these guidelines for free rather than pay design fee for companies.

<u>Guidelines for Designing Antisense Oligos:</u>

1. Predict target RNA secondary structure, then calculate the free energy of the hybrid complex of target RNA and antisense oligo (17 – 20 mers). Oligos generate lower free energy state of hybrid complexes are candidates for effective antisense oligo, because low free energy means the hybrid complex is more stable and easier to form. The program P.I. used is RNAstructure, version 3.6 developed by Dr. Douglas H. Turner, Department of Chemistry, University of Rochester. This program is an implementation of the Turner-Zuker algorithm for RNA secondary structure prediction based on free energy minimization.

It is free and can be download at rna.chem.rochester.edu/RNAstructure.html. Free energy calculation is done by OligoWalk, which comes with RNAstructure.

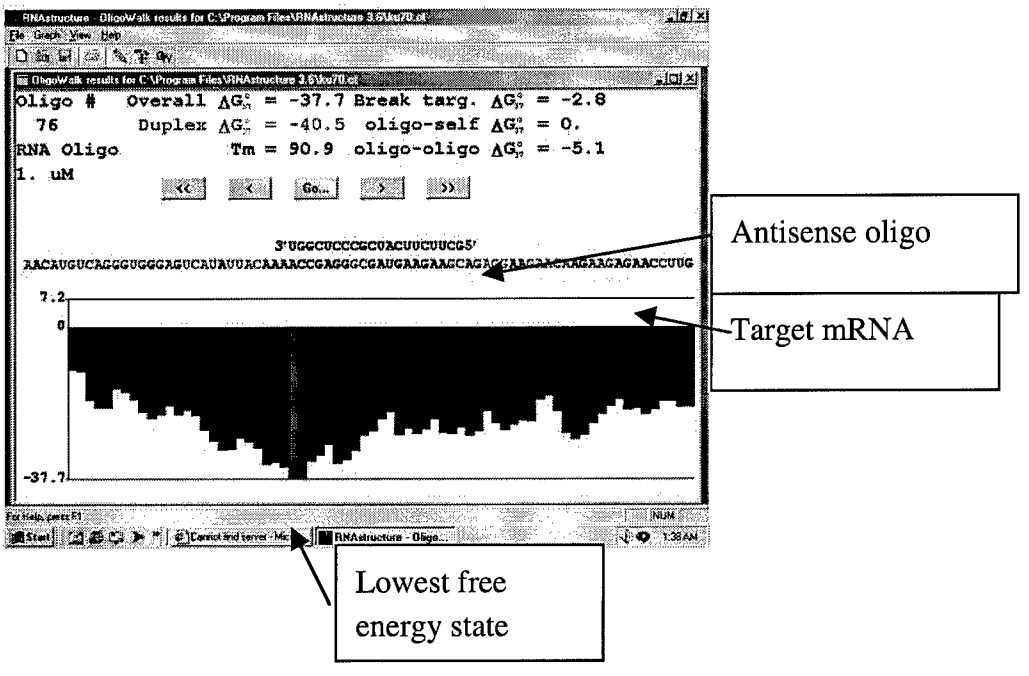
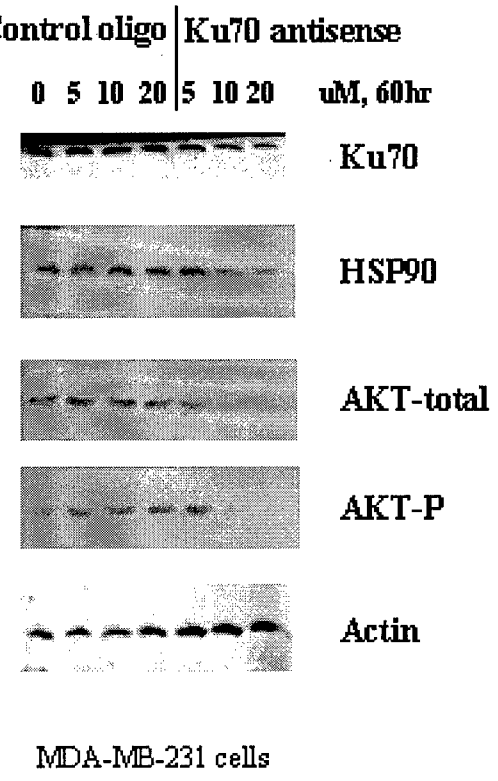


Figure 2. Screen shot of OligoWalk after RNA secondary structure prediction. The antisense oligo used in the following experiments is shown above, which has the lowest free energy state in all (2132) possible hybrid complexes as indicated.

2. Pick antisense candidates with at least 50% GC content.
3. Blast search (NCBI) picked candidates to make sure that they are unique to the target gene.
4. Synthesize antisense oligos with backbone modification to extend the half-life of antisense oligo inside cells. In this case, we use phosphorothioate as backbone, since this kind of modified oligo is in clinical trial now.
5. Antisense oligo can be used to treat cells directly with higher concentration or used with OligofectAMINE (GibcoBRL) in lower concentration.
6. Immuno-western blots for the target gene are used to valid the effectiveness of antisense oligos.

Antisense Treatments

A.



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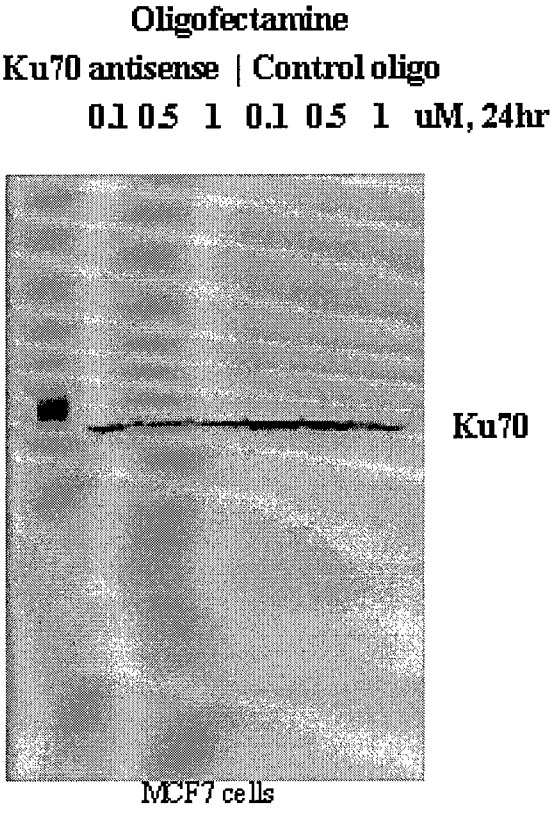

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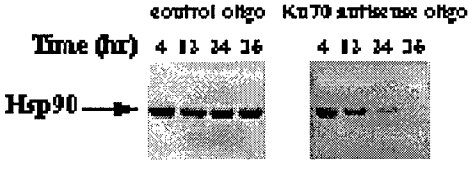

Figure 3. Ku70 Antisense Treatments.

(A) Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense oligo or control oligo (sense oligo) in 5, 10, 20 uM for 60 hours. Ku70 protein expression decreases in 10, 20 uM treatment as validated by immuno-western blot. Hsp90 expression decreases corresponding to Ku70 protein decreases. Akt kinase, one of Hsp90 client proteins, which play a critical role in cell growth and survival was examined. Both Total Akt (Akt-Total) and Phosphorylated or activated Akt (Akt-P) are reduced corresponding to Hsp90 and Ku70 reduction. Total Actin (all isoforms) expression levels are served as loading controls.

(B) Ku70 antisense oligo is effective in human breast cancer MCF7 cells as well. In this experiment, we use OligofectAMINE purchased from GibcoBRL to facilitate intake of antisense oligo in cells, and reduce oligo concentration to 0.5 uM (20 fold lower) and time of treatment to 24 hours with OligofectAMINE compare to oligo alone.

(C)Hsp90 protein half-life was examined by S^{35}-methione pulse trace after Ku70 antisense or control oligo treatment (20 uM). Hsp90 protein was stable over 36 hr in the normal condition. Its half-life was shortened to around 12 hr after Ku70 antisense treatment.

Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense oligo or control oligo as shown in Figure 3A. Ku70 protein expression decreases in 10, 20 uM treatment as validated by immuno-western blot. Hsp90, a new isolated Ku70 associated protein, expression decreases corresponding to Ku70 protein decreases as well (Figure 3A). It is possible that Ku70 and Hsp90 interaction may be needed for Hsp90 protein stability. Blocking Ku70 protein expression shorten the half-life of Hsp90 (Figure 3C), and subsequently affect on Hsp90 client proteins. We further examine Akt kinase, one of Hsp90 client proteins, which play a critical role in cell growth and survival. As shown in Figure 3A, both total Akt (Akt-Total) and Phosphorylated or activated Akt (Akt-P) are reduced corresponding to Hsp90 and Ku70 reduction. Figure 3B shows Ku70 antisense oligo is effective in human breast cancer MCF7 cells as well. In this experiment, we use OligofectAMINE purchased from GibcoBRL to facilitate intake of antisense oligo in cells, and we can reduce oligo concentration to 0.5 uM (20 fold lower) and time of treatment to 24 hours with OligofectAMINE compare to oligo alone. Furthermore, Hsp90 protein half-life was examined by S^{35}-methione pulse trace. After Ku70 antisense oligo treatment (20 uM), Hsp90 half-life was shortened to around 12 hr compared to stable over 36 hr in the normal condition (Figure 3C).

E. Proteomics analysis in protein expression and function level:

P.I. has collaborated with Dr. Shuyuan Yeh, an Assistant Professor at the Department of Urology, University of Rochester to establish Proteomics techniques using 2-dimension electrophoresis. We have setup the Pharmacia Multiphor II IEF System with Immobiline DryStrip for the first dimension (Isoelectric focusing). Isoelectric focusing (IEF) with an immobilized pH gradient (IPG) DryStrip make IEF easier and much more reproducible on the spot distribution along the pH gradient. IPG DryStrip also makes it possible to focus basic proteins in the gel and to obtain distinct protein spots as shown in the following two panels.

Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense or control oligo, 20uM for 60 hr as demonstrated in Figure 3. Protein lysates were separated by 2D electrophoresis using Pharmacia DryStrip (pH 3-10, linear gradient) in Multiphor II IEF System for isoelectric focusing. The second dimension was carried out in regular vertical SDS-PAGE apparatus. The pictures were taken after silver stain (Figure 4A). Reproducible spot distributions of these two gels are observed. Just by comparing each protein spot by naked eyes, we have identified at least 7 spots either up or down expressed after Ku70 antisense treatment without help of fancy Proteomics hardware and software (Figure 4A). With combination of 2D gel and Phospho-specific antibodies (Abs), we can identify protein kinase or substrates that changed after treatment. Here, we demonstrated using phospho-specific Akt substrate antibody to identify at least 5 Akt substrates were dephosphorylated after turning down Ku70 (Figure 4B, the signature pattern for Akt substrates).

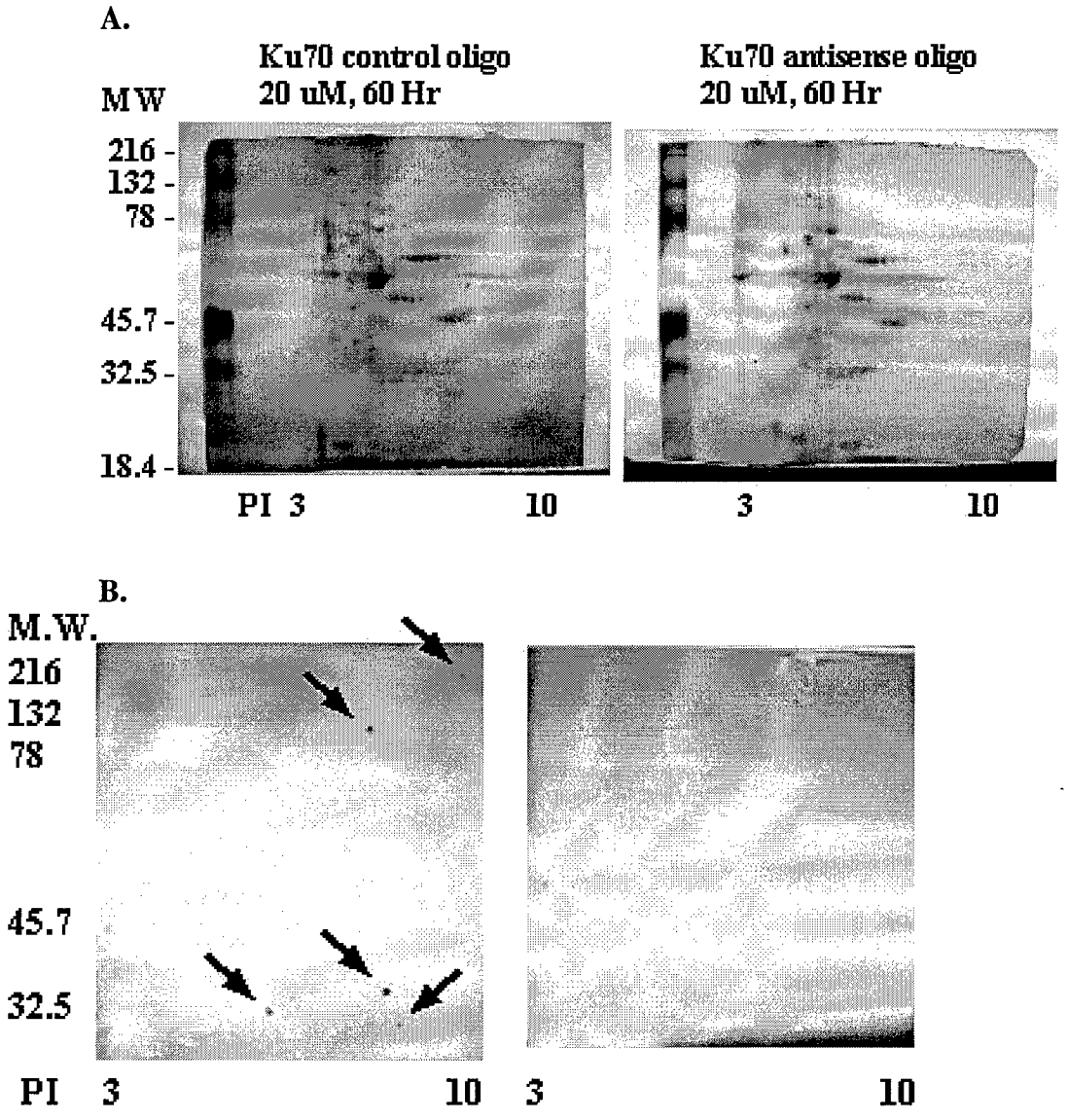

Figure 4. Proteomics analysis of the Ku70 proteomic network.

(A) Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense or control oligo, 20uM for 60 hr, and cell lysates were separated by 2D electrophoresis. The pictures were taken after silver stain. Reproducible spot distributions of these two gels are observed. At least 7 spots either up or down expressed were identified after Ku70 antisense treatment.

(B) The signature pattern for Akt substrates: Using the same cell lysates in (A), after 2D electrophoresis, gels were transferred to PVDF membranes and probed with phospho-specific Akt substrat antibody. De-phosphorylation of at least 5 Akt substrates were observed after Ku70 antisense treatment.

Key Research Accomplishments

- Ku70 - Hsp90 interaction
  - Ku70-Hsp90 interaction in yeast two-hybrid analysis
  - Ku70-Hsp90 interaction in human breast cancer cells by co-immunoprecipitation and colocalization of immuno-staining.
  - Ku70's Hsp90 binding domain is overlapped with Ku70's DNA binding domain.
- Hsp90 and Akt kinase are regulated by the Ku70.
  - Blocking Ku70 protein expression shortened the half-life of Hsp90, and subsequently down-regulated Akt kinase.
  - Proteomic analysis confirmed the de-phosphorylation of at least 5 Akt substrates after Ku70 antisense oligonucleotide treatment.

Reportable Outcome

- Manuscript submitted:

  Title: Ku70 is Critical for Stabilizing Hsp90 and Maintaining Akt Kinase Activity
  Authors: Chin-Rang Yang*, Weimin Liu, Shuyuan Yeh, Ivan Ding, Paul Okunieff
  * Corresponding Author

- Patent pending

  Title: Disruption of Ku70 and Hsp90 protein interaction
  Inventors: Chin-Rang Yang, Paul Okunieff

Conclusions

Our hypothesis is that Ku70 is a target for cancer therapy. Understanding and blocking Ku70 mediated - DNA repair and cell growth pathway can enhance chemo- and radio- therapies for cancers in one treatment. Understanding the downstream cell growth pathways mediated by Ku70 (e.g. Akt kinase pathway) will be very helpful to develop a combination therapy.

We found that Hsp90 (heat shock protein 90) and Akt kinase are regulated by the Ku70. The eukaryotic Hsp90 is a molecular chaperone and its client proteins are typically involved in signal transduction, cell cycle control, or transcriptional regulation (4,5). We confirmed Ku70-Hsp90 interaction in yeast two-hybrid analysis as well as in cancer cells, which occurs predominately in the mitotic phase demonstrated by co-immunoprecipitation and colocalization of immuno-staining. Ku70's Hsp90 binding domain is overlapped with Ku70's DNA binding domain. Blocking Ku70 protein expression using an antisense oligonucleotide shortened the half-life of Hsp90, and subsequently down-regulated Akt kinase. Using 2-dimension electrophoresis and immuno-western blot with phospho motif

-specific antibodies, we further confirmed the de-phosphorylation of at least 5 Akt downstream substrates after Ku70 antisense oligonucleotide treatment, which is consistent with loss of Akt kinase. Taken together, our results suggest that Ku70-Hsp90-Akt pathway is one of the Ku70 mediated cell growth pathways, and Ku70-Hsp90 protein complex is a novel drug target for future cancer therapy.

Overall, the research supported by this grant may not only lead to a better understanding of factors involving in Ku70-mediated cell growth pathways, but also lead to the identification of factors which can sensitize tumor cells to chemo- and radio- therapies in one treatment.

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Appendices

- Manuscript submitted
- Letter for US Patent Provisional Application

Title: Ku70 is Critical for Stabilizing Hsp90 and Maintaining Akt Kinase Activity

Authors: Chin-Rang Yang, Weimin Liu, Shuyuan Yeh*, Ivan Ding, Paul Okunieff

Author Affiliation: Department of Radiation Oncology

Department of Urology*

University of Rochester

Corresponding Author: Chin-Rang Yang, PhD

Short Title: Ku70 Regulates Hsp90 and Akt

Key Words: Ku70, Hsp90, Akt, DNA Repair, Cell Signaling

Summary

Ku70 is a protein involved in DNA double strand break repair. However, Ku70 knock-out mice show not only hypersensitive to ionizing radiation, but also growth retardation. We found that Hsp90 (heat shock protein 90) and Akt kinase are regulated by the Ku70 mediated cell growth pathway. The eukaryotic Hsp90 is a molecular chaperone and its client proteins are typically involved in signal transduction, cell cycle control, or transcriptional regulation. We confirmed Ku70-Hsp90 interaction in yeast two-hybrid analysis as well as in cancer cells, which occurs predominately in the mitotic phase demonstrated by co-immunoprecipitation and colocalization of immuno-staining. Ku70 protein expression was then blocked using an antisense oligonucleotide designed by the Turner-Zuker algorithm for RNA secondary structure prediction and oligonucleotide hybridization based on free energy minimization. Blocking Ku70 protein expression shortened the half-life of Hsp90, and subsequently down-regulated Akt kinase. Using 2-dimension electrophoresis and immuno-western blot with phospho motif -specific antibodies, we further confirmed the de-phosphorylation of at least 5 Akt downstream substrates after Ku70 antisense oligonucleotide treatment, which is consistent with loss of Akt kinase. Taken together, our results suggest that Ku70-Hsp90-Akt pathway is one of the Ku70 mediated cell growth pathways.

Introduction

The DNA-dependent protein kinase (DNA-PK) complex is a well-known factor in the repair of DNA double strand breaks (DSBs) in mammalian cells. DNA-PK is a multi-protein complex composed of a DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs, 460 kDa) and a DNA end binding subunit, the Ku autoantigen [a nuclear heterodimer of 70 kDa (Ku70) and 80 kDa (Ku80) proteins] (Tuteja, 2000). Interestingly, Ku70 knock-out mice show not only hypersensitive to IR, but also growth retardation (Gu, 1997). In contrast, DNA-PKcs knock-out mice show hypersensitive to IR but have normal growth rate (Gao, 1998). The results indicate that Ku70 is not only involved in DNA repair but also in cell growth, and the cell growth function is independent of DNA-PK function. To further explore the Ku70 mediated cell growth pathway, we applied the Yeast-Two Hybrid Analysis to identify new Ku70 Associated Proteins (Yang, 1999)(Yang, 2000).

One of Ku70 associated proteins identified is heat shock protein 90 (Hsp90). The eukaryotic Hsp90 is one of molecular chaperones playing a key role in protein folding, activation and assembly. Its client proteins are typically involved in signal transduction, cell cycle control, or transcriptional regulation, including Raf, AKT, the Her2/ErbB receptor family, Wee1, steroid receptors (Pearl, 2000), nitric oxide synthase, and telomerase (Caplan, 1999). Hsp90 consist of a highly conserved ~25 kDa N-terminal domain with an ATP binding pocket and ~55 kDa C-terminal dimerization domain. Two Hsp90s dimerize through their C-terminus and binding of ATPs in their N-terminus cause a conformation change to bring their N-terminus close together. This mechanism is called "ATPase-coupled molecular clamps" and is thought to be important for the proper function of Hsp90's client protein (Pearl, 2000). The ability of Hsp90 to simultaneously interact with multiple oncogenic proteins makes it the potential drug target for the future cancer therapy (Workman, 2002). Here, we demonstrate that not only Ku70-Hsp90-Akt pathway is one of Ku70 mediated cell growth

pathways, but also the interaction with Ku70 is important for the stability of Hsp90. Therefore, targeting Ku70-Hsp90 interaction may have profound effects on killing cancer cells.

Materials and Methods

Yeast Two-Hybrid Analysis

The Yeast Two-Hybrid System was generously provided by S. Elledge (Baylor College of Medicine, Houston, TX). The detail procedures were described elsewhere (Yang, 1999)(Yang, 2000). The fresh prepared medium containing 25 mM 3AT (3-amino-1,2,4-triazole) to inhibit leaky histidine synthatase (HIS3) is the key to suppress non-specific positive clones. In this condition, the interaction of Ku70 and Hsp90 in yeast was tested repeatedly with other negative controls (Table 2) to insure the interaction is specific.

The Hsp90 we cloned out is also called Hsp90 alpha, the GenBank accession number is X15183 or M27024.

Cell Culture and Treatments

Human breast cancer MCF7 and MDA-MB-231 cells were purchased from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS in a 37 degree, 5%CO2 incubator. Colcemid (GibcoBRL, KaryoMax solution, 100ng/ml) was used to arrest cells in G2/M phase. Geldanamycin (Sigma, G-3381, 20 uM), a known Hsp90 inhibitor, was used to block Ku70-Hsp90 interaction.

Antibodies

Mouse monoclonal antibody to human Ku70 (N3H10) was obtained from Dr. R. Burgess (Univ. of Wisconsin-Madison). Goat polyclonal antibodies to human Hsp90 (N-17, sc-1055) and actins (I-19,

sc-1616) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to total Akt (#9272), phosphorylated Akt (Ser473, #9271), and phospho-Akt substrates (#9611S) were purchased from New England Biolabs, inc.

Co-immunoprecipitation and Co-localization

Cell lysates were prepared as described (Yang, 1999). Coimmunoprecipitation and immuno-cytochemical stain for confocal microscopy were performed as described (Yang, 2000) with Hsp90 antibody.

S^{35}-Methionine Pulse Trace for Hsp90 Half-Life

The protocol was described in detail elsewhere (Spector, 2000). Briefly, cells were treated with Ku70 antisense or control oligonucleotides, then pulse labeled with S^{35}-Methionine (TRAN ^{35}S-LABEL with 70% methionine, 30% cysteine purchased from ICN) 5 uCi/ml for 3 hours in CELLect Deficient DMEM without L-Methionine (ICN) + 10% dialyzed fetal bovine serum. Cells lysates taken at the different time points were immunoprecipitated by Hsp90 antibody, run on SDS-PAGE, and imaged by PhosphoImager (Molecular Dynamics).

Antisense Oligonucleotide Design and Treatment

Antisense oligonucleotide was used to block Ku70 mRNA translation and protein expression. However, due to the secondary structure of mRNA, designing an effective antisense oligonucleotide is a challenge. Our strategy is to first predicted Ku70 RNA secondary structure, then calculate the free energy states of all possible hybrid complexes of Ku70 RNA and antisense oligonucleotides (20 mers). Oligonucleotides ,which form hybrid complexes with target mRNA in lower free energy states, are candidates for antisense treatment, because they produce rapidly forming hybrid complexes that are more stable. The

program we used is RNAstructure, version 3.6 developed by Dr. Douglas H. Turner, Department of Chemistry, University of Rochester. This program is an implementation of the Turner-Zuker algorithm for RNA secondary structure prediction based on free energy minimization. It is free for download at http://rna.chem.rochester.edu/RNAstructure.html. Free energy calculations of hybrid complexes were done by OligoWalk, which included in RNAstructure. The antisense oligonucleotide used in our experiments has the lowest free energy state in all (2132) possible hybrid complexes. Blast search (NCBI) indicated that it is unique to human Ku70. The antisense oligonucleotide was synthesized with backbone modification (phosphorothioate) to extend its half-life inside cells. The antisense oligonucleotide was used to treat cells either directly or with OligofectAMINE (GibcoBRL). Immuno-western blots for Ku70 are used to validate the effectiveness of antisense oligonucleotides. The sequence of oligonucleotides are: antisene oligonucleotide: 5'- CGTTGAAGTACGCCCACGGA –3'; control (Sense) oligonucleotide: 5'- GCAACTTCATGCGGGTGCCT –3'

2D Gel and Phospho Akt substrates detection

Protein lysates were separated by 2D electrophoresis using Pharmacia DryStrip (pH 3-10, linear gradient, 11 cm) in Multiphor II IEF System for isoelectric focusing per manufacturer's instruction. The composition of the protein lysis buffer is 8M Urea, 2%CHAPS, 2%IPG buffer, 2.8 mg/ml DTT, and trace Bromophenol blue. The second dimension was carried out in regular vertical SDS-PAGE apparatus. After the second run, gels were either silver stained or transferred to PVDF membranes probing with phospho-specific Akt substrate antibody.

Results

Isolation of Ku70 Associated Proteins in the Yeast Two-Hybrid Analysis

The Yeast Two-Hybrid clonings were performed using human Ku70 as bait and human bone marrow, ovary, and liver cDNA libraries as prey. The known Ku70 associated proteins, Ku80 and clusterin, were isolated as expected (Table 1). The new Ku70 associated protein is Hsp90, and was cloned using the bone marrow library (Table 1). Hsp90 interacts specifically with Ku70, not with Ku70 StuI (a mutant Ku70 with c-terminus deletion), Ku80, p53, cdk2, lamin B and SNF-1 (Table 2). The negative control, Hsp70 (heat shock protein 70) did not interact with Ku70 (Table 2). Thus, we conclude that Ku70 and Hsp90 interaction is specific in the Yeast Two-Hybrid Analysis.

Ku70 StuI mutant is a partial C-terminus deletion of the Ku70 DNA binding domain (573-607 a.a.), which is called the SAP domain (Aravind, 2001). The SAP domain is a putative DNA-binding motif (alpha helix – turn – alpha helix structure) that is also found in several other DNA binding proteins. The result (Table 2) indicates that the DNA and Hsp90 binding domain of Ku70 may be overlapping.

Interaction of Ku70 and Hsp90 in human Cancer Cells

Since Ku70 is primarily located in nuclei and Hsp90 is primarily located in cytoplasm, we theorize that these two proteins may meet during the mitotic phases of cell cycle when the nuclear membrane is absent. We first treated MCF-7 cells with colcemid (100ng/ml) for 18 hours. Under these conditions, over 80% of cells are in G2/M measured by flow cytometry. Cell lysates were harvested and co-immunoprecipitaion was performed using antibodies as indicated in Figure 1A. In Figure 1A, lane 4 indicates that more Ku70/Hsp90 complexes were formed when cells are in G2/M phase than untreated cells (lane 3). Normal IgG (lane 1) and Hsp70 antibody (lane 2) immunoprecipitation did not bring down Ku70 and served as negative controls for the specificity of

Ku70-Hsp90 interaction. Geldanamycin (GM), an Hsp90 inhibitor that binds Hsp90 (Srethapakdi, 2000), blocked the interaction of Ku70 and Hsp90 (lane 5) possibly due to the conformation change of Hsp90 after GM binding. In Figure 1B, most of cells show distinct distribution of Ku70 (in nuclei, red) and Hsp90 (in cytoplasm, green). However, cells in the mitotic phase of the cell cycle showed colocalization of these two proteins (indicated by white arrowheads). To further understand the function of the Ku70-Hsp90 interaction and its impact on the Ku70 mediated cell growth pathway, we decide to use a designed Ku70 antisense oligonucleotide to block Ku70 protein expression.

Design of Antisense Oligonucleotide

Antisense oligonucleotides function by hybridizing to their target mRNA; thereby, blocking translation. However, due to the secondary structure of mRNA, designing an effective antisense oligonucleotide is a challenge. To create an effective oligonucleotide that blocks Ku70 synthesis, we first predicted the Ku70 mRNA structure by "RNAstructure 3.6" and "OligoWalk" developed by Dr. Douglas H. Turner, Department of Chemistry, University of Rochester. These programs are based on the Turner-Zuker algorithm for RNA secondary structure prediction and minimize the free energy for oligonucleotide hybridization. The antisense oligonucleotide used in our experiments has the lowest free energy state in all (2132) possible hybrid complexes, and is specific for human Ku70 according to Blast search (NCBI).

Alteration of Hsp90 and Akt Protein Expression after Ku70 Antisense Oligonucleotide Treatment

Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense oligonucleotide or control oligonucleotide as shown in Figure 2A. Ku70 protein expression decreases after 10 or 20

uM treatment as validated by immuno-western blot. Hsp90 expression decreases corresponding to Ku70 protein decreases as well (Figure 2A). We measured Hsp90 half-life using S^{35}-methionine pulse trace and Hsp90 immunoprecipitation. Hsp90 half-life was shortened to around 12 hr after Ku70 antisense oligonucleotide treatment (Figure 2C), which suggests Ku70 and Hsp90 interaction is needed for Hsp90 protein stability. We further examine Akt kinase, a Hsp90 client protein, which play a critical role in cell growth and survival. As shown in Figure 2A, both total Akt (Akt-Total) and Phosphorylated or activated Akt (Akt-P) are reduced corresponding to Hsp90 and Ku70 reduction. The results are consistent with treatment of Hsp90 inhibitors (herbimycin A and geldanamycin, which bind to the ATP binding pocket of the N-terminus of Hsp90) that alter the function of Hsp90 and result in the degradation of Hsp90's client proteins (Srethapakdi, 2000). Figure 2B shows Ku70 antisense oligonucleotide is effective in human breast cancer MCF7 cells as well. In this experiment, we use OligofectAMINE purchased from GibcoBRL to facilitate intake of antisense oligonucleotide in cell. Using this approach, the effective concentration was reduced 20 fold to 0.5 uM, and the effective treatment time was reduced to 24 hours. Taken together, our results confirm that Ku70-Hsp90-Akt pathway is one of the Ku70 mediated cell growth pathways.

Dephosphorylation of Akt kinase protein substrates after Ku70 Antisense Oligonucleotide Treatment

The same protein lysates in Figure 2 were separated by 2D electrophoresis using Pharmacia DryStrip (pH 3-10, linear gradient) in Multiphor II IEF System for isoelectric focusing. The second dimension was carried out in regular vertical SDS-PAGE apparatus. The pictures were taken after silver stain (Figure 3A). Reproducible spot distributions of these two gels are observed. There are at least 7 protein spots altered after Ku70 antisense treatment (Figure 3A). With combination of 2D gel and Phospho motif -specific antibodies (Abs), we can identify Akt kinase substrates that changed

after Ku70 antisense treatment. Here, using phospho-specific Akt substrate antibody, we demonstrated at least 5 Akt substrates were dephosphorylated after suppressing Ku70 (Figure 3B). Thus, we conclude that Ku70 play an important role in maintaining Hsp90 stability, and the function of Hsp90's client protein, Akt. Ku70-Hsp90 interaction is essential for proper cell growth.

Discussion

We demonstrate that Ku70 is not only a DNA repair protein, but also a stabling factor for Hsp90. Without Ku70, Hsp90 becomes unstable and subsequently affects function of its client proteins, which play important roles in various physiological processes such as signal transduction, cell cycle control, and transcriptional regulation. Based on observations of growth-retarded Ku70 knockout mice, we believe that Ku70 is an essential factor for normal cell growth, and the results of this paper provide one possible mechanism for regulation of the Ku70 mediated cell growth pathway. However, since Hsp90 is not the only Ku70 associated proteins, and Akt is not the only Hsp90 client protein, it is reasonable to expect that other pathways are also involved. We demonstrated that Ku70-Hsp90 protein complex formation is the key for Hsp90 stability and proper function of Hsp90 client proteins, although we do not know the mechanism of Hsp90 degradation. To further complicate the issue, Hsp90 is a co-chaperone and therefore must work with other co-chaperones to serve its client proteins. Several different chaperone complexes have already been identified. For example, two complexes associated with maturation of steroid hormone receptors are Hsp90-Hop(Sti1)-Hsp70-Hip, and Hsp90-p23/immunophilin (Caplan, 1999). The effect of Ku70 knockout on those chaperone complexes is unknown.

Although we demonstrated that Ku70 and Hsp90 interact mainly in the mitotic phase of growing cells, it is possible that Hsp90 may be a chaperone for newly synthesized Ku70 during

nuclear translocation. Under stress conditions when nuclei are damaged such as pathological condition, drugs treatment, or ionizing radiation treatment, Ku70 may leak out to further support Hsp90 function and protect cells against stress. Furthermore, Hsp90 is a new drug target for cancer therapy due to its ability to simultaneously interact with multiple oncogenic proteins (Workman, 2002). For example, anti-tumor agents, ansamycin antibiotics (geldanamycin and herbimycin-A), are known to significantly decrease the activities of oncogenic tyrosine kinases, such as v-Src and ErbB-2. Recent studies suggest that these drugs act by targeting Hsp90, not tyrosine kinases themselves. The drug activities result from disruption of Hsp90 ATP binding and subsequently, Hsp90-dependent tyrosine kinase activities (Pearl, 2000). Geldanamycin has entered the Phase I trial for cancer therapy (Pamela, 2001). Given the Ku70-Hsp90 interaction is important for the stability of Hsp90, targeting the Ku70-Hsp90 interaction should have profound effects on augmenting the killing of cancer cells. Therefore, the Ku70-Hsp90 protein complex could be a new target for cancer therapy.

Moreover, in Table 2, we demonstrated that the Ku70's DNA and Hsp90 binding domain may be overlapping in the region called SAP domain. It is possible that inhibitors targeting the SAP domain (Aravind, 2001) may not only interfere with the Ku70-Hsp90 interaction, but also interfere with the Ku70 DNA binding activity. Thus, SAP domain inhibitors may have dual functions: by blocking both Ku70 DNA binding (DNA repair) and the Ku70-Hsp90 complex (Cell Growth), it should sensitize cancer cells to DNA damage agents (chemotheraputic drugs or ionizing radiation), and by suppressing cancer cell growth by down-regulation of Ku70 function.

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Figure Legends

Figure 1. Ku70-Hsp90 Interaction in mammalian cells.

Human breast cancer MCF-7 cells were used in these experiments.

(A) Lane 1: cell lysate were immunoprecipitated using normal IgG as negative controls;

Lane 2: cell lysate were immunoprecipitated using Hsp70 antibody as negative controls;

Lane 3: cell lysate without any treatment was immunoprecipitated using Hsp90 antibody;

Lane 4: cell lysate with colcemid (100 ng/ml) treatment were immunoprecipitaed using Hsp90 antibody;

Lane 5: cell lysate with Geldanamycin (GM, 20 uM, a Hsp90 inhibitor) treatment were immunoprecipitaed using Hsp90 antibody.

(B) Cells were fixed and immuno-stained with both Ku70 and Hsp90 antibodies. Ku70 antibody was detected by secondary antibody with Texas red (red); and Hsp90 antibody was detected by secondary antibody with FITC (green). Co-localization of these two proteins was visualized by confocal microscopy and shown in yellow color (white arrowheads).

Figure 2. Alteration of Hsp90 and Akt after Ku70 Antisense Treatment.

(A) Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense oligonucleotide or control (sense) oligonucleotide at 5, 10, or 20 uM for 60 hours. Ku70 protein expression decrease after either 10 or 20 uM treatment as validated by immuno-western blot. Hsp90, the newly isolated Ku70 associated protein, decreased along with Ku70 protein, suggesting that Ku70 may be needed for Hsp90 protein stability. Akt kinase, one of the Hsp90 client proteins, plays an important role in cell growth and survival. Both total Akt (Akt-Total) and Phosphorylated or activated Akt (Akt-P) protein expressions are reduced along with Hsp90 and Ku70. Total actin (all isoforms) expression levels served as loading controls.

(B) Ku70 antisense oligonucleotide is effective in human breast cancer MCF7 cells. OligofectAMINE (GibcoBRL) can facilitate uptake of antisense oligonucleotide into cells, and can reduce the effective oligonucleotide concentration 20-fold to 0.5 uM and the effective treatment time to 24 hours.

(C) Hsp90 protein half-life was examined by S^{35}-methione pulse trace. S^{35}-methione labeled Hsp90 was immunoprecipitaed by Hsp90 antibody after Ku70 antisense or control oligonucleotide treatment (20 uM) in MDA-MB-231 cells. Hsp90 protein was stable over 36 hr in the control condition. Its half-life was shortened to around 12 hr after Ku70 antisense oligonucleotide treatment.

Figure 3. Proteomic analysis of Akt kinase substrates after Ku70 Antisense Oligonucleotide Treatment.

(A) Human breast cancer MDA-MB-231 cells were treated with Ku70 antisense or control oligonucleotide, 20uM for 60 hr, and cell lysates were separated by 2D electrophoresis. The pictures were taken after silver stain. Reproducible spot distributions of these two gels are observed.

(B) Using the same cell lysates in (A), after 2D electrophoresis, gels were transferred to PVDF membranes and probed with phospho-specific Akt substrate antibody. Dephosphorylation of at least 5 Akt substrates (indicated by arrows) were observed after Ku70 antisense treatment.

Table 1: Ku70-associated Proteins by Yeast Two-hybrid Analysis

	Gene	Human cDNA library
1.	Ku80	Liver, Bone Marrow, Ovary
2.	Clusterin	Liver, Bone Marrow, Ovary
3.	Heat Shock Protein 90	Bone Marrow
4.	unknown	Liver, Bone Marrow, Ovary

Table 2: Specificity of Ku70-Hsp90 Interaction

	Hsp90	Hsp70
Ku70	++	--
Ku70 StuI	--	--
Ku80	--	--
p53	--	--
cdk2	--	--
lamin B	--	--
SNF-1	--	--

++ : interaction was verified by Histidine selection

and beta-galactosidase activity

__: no interaction

Figure 1

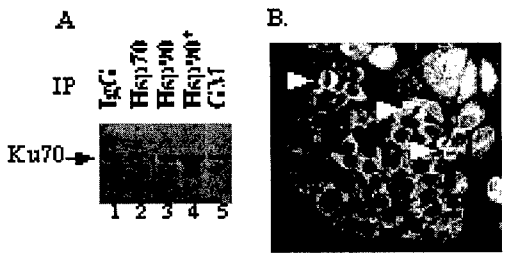


Figure 2

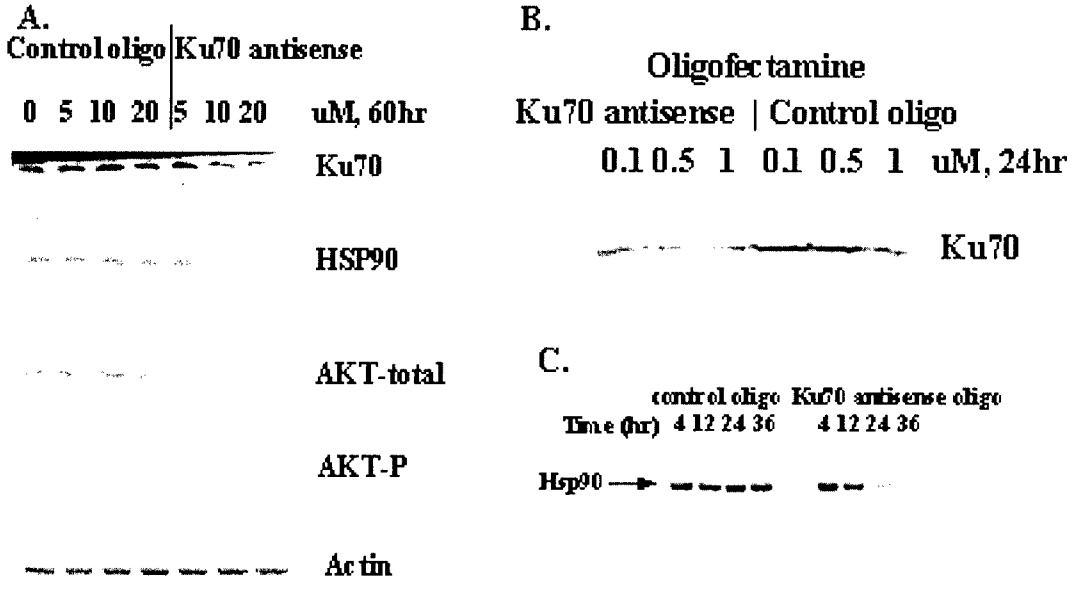
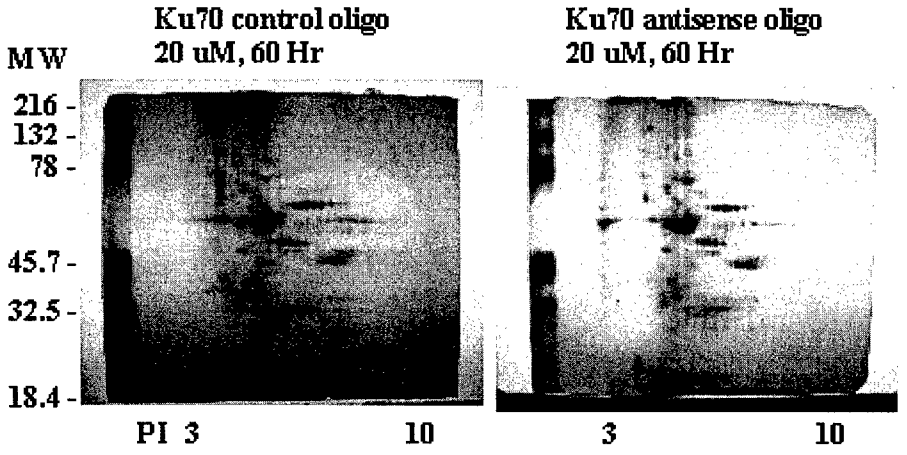

A.
Control oligo
Ku70 antisense
0 5 10 20 5 10 20 uM, 60hr
Ku70
HSP90
AKT-total
AKT-P
Actin
B.
Oligofectamine
Ku70 antisense | Control oligo
0.1 0.5 1 0.1 0.5 1 uM, 24hr
Ku70
C.
control oligo
Ku70 antisense oligo
Time (hr) 4 12 24 36 4 12 24 36
Hsp90

Figure 3

A.


Ku70 control oligo
20 uM, 60 Hr
Ku70 antisense oligo
20 uM, 60 Hr
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132 -
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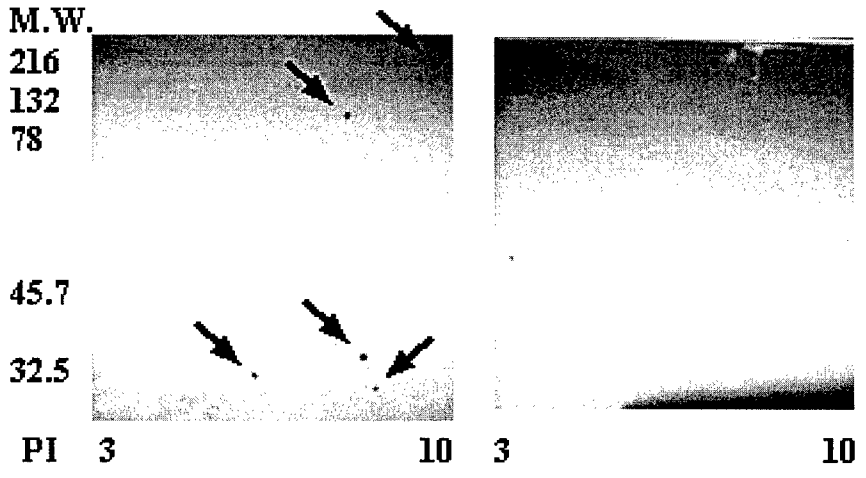
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Jules E. Goldberg ▪ 212.521.5403 ▪ jgoldberg@reedsmith.com

March 27, 2002

Your Ref: Tech I.D. #1092
Our Ref: JG-RMC-5125PRO/500954.20002

Dr. Chin-Rang Yang
Radiation of Oncology
University of Rochester Medical Center
601 Elmwood avenue, Box 647
Rochester, NY 14642

Re: New U.S. Provisional Application
In the name(s) of Dr. Chin-Rang YANG and Dr. Paul OKUNIEFF
Entitled: DISRUPTION OF Ku70 AND Hsp90 PROTEIN INTERACTION

Dear Dr. Yang:

We thank you for your letter of March 14, . We have filed the above -identified Provisional Application with the U.S. Patent and Trademark Office today. Enclosed is a copy of the application as filed.

We can expect to receive an Official Filing Receipt in due course and shall keep you promptly advised with respect thereto.

Please note that if this application is to be pursued, a non-provisional application must be filed on or before:

March 27, 2003.

Please note that also by this date, any non-U.S. filings must be affected in order to retain the benefit of the U.S. filing date.

Thank you and Dr. Okunieff for your assistance in preparation of this application and I look forward to working with you on this and other matters in the future.

Kind regards.

Very truly yours,

Jules E. Goldberg

JEG:dej

cc: Dr. Paul Okunieff
Dr. Claudia Stewart

375 Park Avenue
17th Floor
New York, NY 10152-1799
212.521.5400
Fax 212.521.5450

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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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Deputy Chief of Staff for
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